HIGHLIGHTED TOPIC | Lung Growth and Repair

Hypoxia-responsive growth factors upregulate peristin and osteopontin expression via distinct signaling pathways in rat pulmonary arterial smooth muscle cells

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Li, Peng, Suzanne Oparil, Wenguang Feng, and Yiu-Fai Chen. Hypoxia-responsive growth factors upregulate peristin and osteopontin expression via distinct signaling pathways in rat pulmonary arterial smooth muscle cells. J Appl Physiol 97: 1550–1558, 2004. First published April 30, 2004; 10.1152/japplphysiol.01311.2003.—This study tested the hypothesis that expression of the novel adhesion molecule peristin (PN) and osteopontin (OPN) is increased in lung and in isolated pulmonary arterial smooth muscle cells (PASMCs) in response to the stress of hypoxia and explored the signaling pathways involved. Adult male rats were exposed to 10% O2 for 2 wk, and growth-arrested rat PASMCs were incubated under 1% O2 for 24 h. Hypoxia increased PN and OPN mRNA expression in rat lung. In PASMCs, hypoxia increased PN but not OPN expression. The hypoxia-responsive growth factors fibroblast growth factor-1 (FGF-1) and angiotensin II (ANG II) caused dose- and time-dependent increases in PN and OPN expression in PASMCs. FGF-1-induced PN expression was blocked by the FGF-1 receptor antagonist PD-166866 and by inhibitors of phosphatidylinositol 3-kinase (PI3K) (LY-294002, wortmannin), p70S6K (rapamycin), MEK1/2 (U-0126, PD-98059), and p38MAPK (SB-203580) but not of JNK (SP-600125). ANG II-induced PN expression was blocked by the AT1-receptor antagonist losartan and by inhibitors of PI3K and MEK1/2. In contrast, FGF-1-induced OPN expression was blocked by inhibitors of JNK or MEK1/2 but not of PI3K, p70S6K, or p38MAPK. Activation of p70S6K and p38MAPK by anisomycin robustly stimulated PN but not OPN expression. This study is the first to demonstrate that growth factor-induced expression of PN in PASMCs is mediated through PI3K/p70S6K, Ras/MEK1/2, and Ras/p38MAPK signaling pathways, whereas the expression of OPN is mediated through Ras/MEK1/2 and Ras/JNK signaling pathways. These differences in signaling suggest that PN and OPN may play different roles in pulmonary vascular remodeling under pathophysiological conditions.

We have recently reported that expression of peristin [PN, a putative bone extracellular matrix (ECM) protein previously named osteoblast-specific factor-2 (5, 6, 10, 18, 23)] is greatly increased during remodeling of the cardiovascular system under stressful conditions, e.g., pressure overload-induced cardiac hypertrophy and balloon-induced endoluminal injury of carotid arteries (12, 26). PN mRNA expression was increased 12- to 43-fold in hearts of wild-type and atrial natriuretic peptide-deficient mice, respectively, with transverse aortic constriction-induced cardiac hypertrophy. This was the most dramatic response to transverse aortic constriction-induced pressure overload of any of >5,000 genes studied by microarray analyses (26). Immunohistochemical analyses showed that PN is preferentially localized to the cardiac fibroblasts, interstitium, and vascular smooth muscle cells (VSMCs) in these experimental settings. Importantly, increased PN expression was associated with increased expression of osteopontin (OPN), collagens, fibronectin, thrombospondin, and matrix metalloproteinases (12, 26), suggesting that PN may participate in ECM remodeling in heart and blood vessels under stressful conditions (26).

The present study expanded these important findings to examine the regulation of PN and OPN in lungs of hypoxia-adapted rats and pulmonary arterial smooth muscle cells (PASMCs) exposed to hypoxia or hypoxia-responsive growth factors in vitro. Based on our findings in the heart and our previous experience with pulmonary vascular remodeling in the lung of hypoxia-adapted rats, we postulated that PN and OPN expression would increase as part of a generalized ECM response to hypoxic stress in the lung of adult rats and in PASMCs in vitro (12, 26). Accordingly, this study tested the primary hypothesis that PN and OPN are increased in lung and in isolated PASMCs in response to normobaric hypoxia exposure. We further tested whether hypoxia-induced growth factors, including the tyrosine kinase receptor-associated growth factor fibroblast growth factor-1 (FGF-1) (13) and the G-protein-coupled receptor-associated growth factor angiotensin II (ANG II) (19), stimulate increased expression of ECM molecules in PASMCs. Finally, we used selective kinase inhibitors to delineate the signaling pathways involved in FGF-1- and ANG II-induced PN and OPN expression in PASMCs.

MATERIALS AND METHODS

Animals and cell culture. Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, DE) at age 8 wk. All animals used in this study were cared for and handled according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. For in vivo chronic hypoxia studies, rats were exposed to hypoxia (10% O2, 1 atm) in an 800-liter model 818GBB Plexiglas glove box (Plas Labs, Lansing, MI) beginning at age 8–9 wk for a total of 2 wk as previously described (3, 4, 21).
For in vitro studies, PASMCs were initially grown in DMEM (GIBCO BRL, Rockville, MD) with 10% heat-inactivated low-endotoxin FBS (GIBCO BRL), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 95% air-5% CO₂ and were passaged by harvesting with trypsin-EDTA and seeding in 6-cm cell culture dishes. Passaged cells were grown in DMEM with 10% FBS. PASMCs were used for experiments between passages 4 and 8.

To confirm the characteristics of smooth muscle cells in culture, immunohistochemical staining of α-actin was performed with specific α-actin antibody and peroxidase-labeled anti-IgM or anti-IgG antibodies.

Before each study, PASMCs were grown to 100% confluence and then made quiescent by placing them in medium containing 0.1% FBS for 24 h. To examine the effects of hypoxia on PN and OPN mRNA expression, PASMCs were transferred into an air-tight hypoxic chamber (model 2710 cell culture incubator, Queue Systems) containing 1% O₂-5% CO₂-94% N₂ as previously described (13, 22). For the study of signal transduction pathways, PASMCs were pretreated with selective inhibitors for 45 min before addition of growth factors (FGF-1 or ANG II) to the medium and incubation for an additional 24 h. For treatment with FGF-1, heparin was added to the medium at a final concentration of 5 U/ml because it protects FGF-1 against enzymatic digestion and promotes FGF receptor binding (28).

Immunohistochemical analysis of PN in lung. At 2 wk after initiation of hypoxic exposure, the lungs from hypoxia-adapted and air control rats were fixed in the distended state by infusion of 10% buffered formalin into the pulmonary artery and trachea at 100 and 25 mmHg pressure, respectively, for 1 min, and then placed in a bath of 4% paraformaldehyde for 24 h, paraaffin embedded, and sectioned for immunohistochemical examination of PN as previously described (4, 26).

RNA extraction and Northern blot analysis. Lungs from 2-wk hypoxia-adapted or air control rats or cultured PASMCs were homogenized, and total RNA was extracted with the use of the TRIZOL total RNA extraction reagent (GIBCO BRL) according to the manufacturer's instructions. Total RNA from each lung or plates of PASMCs was quantified by electrophoresis in denaturing formaldehyde-agarose gels and stained with ethidium bromide. The Northern blot membrane was probed with PN and OPN cDNAs and 18S rRNA oligonucleotide sequentially. The mRNA data were normalized by 18S rRNA to correct for variations in RNA loading. Results are means ± SE. *P < 0.05 vs. respective air or normoxia control groups by unpaired t-test (in vivo study) or 1-way ANOVA (in vitro study).

Fig. 1. A and B: effects of 2-wk hypoxic exposure (10% O₂, 1 atm) on steady-state periostin (PN) and osteopontin (OPN) mRNA expression in rat lung in vivo. Air controls were rats exposed to room air during the same period. C and D: effects of 6- to 24-h hypoxic exposure (1% O₂) on steady-state PN and OPN mRNA expression in pulmonary arterial smooth muscle cells (PASMCs) in vitro. Growth-arrested PASMCs were exposed to hypoxia for 6, 12, and 24 h before being harvested. Normoxic controls were PASMCs cultured in 0.1% FBS medium for 24 h and exposed to 21% O₂ for an additional 24 h. Numbers in parentheses are the number of rats or plates of PASMCs contributing data to each group. Northern blot analysis was carried out with 5 μg of total RNA extracted from each lung or plates of PASMCs. The Northern blot membrane was probed with PN and OPN cDNAs and 18S rRNA oligonucleotide sequentially. The mRNA data were normalized by 18S rRNA to correct for variations in RNA loading. Results are means ± SE. *P < 0.05 vs. respective air or normoxia control groups by unpaired t-test (in vivo study) or 1-way ANOVA (in vitro study).

Fig. 2. Representative light micrographs of small pulmonary arteries (PA; ~100 μm) from rats exposed to chronic hypoxia (10% O₂) for 2 wk or room air. Lung sections were immunostained (brown stain) with selective rabbit anti-PN polyclonal antibody. Sections were counterstained with hematoxylin and eosin. BR, bronchi.

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Fig. 3. Dose- and time-dependent effects of fibroblast growth factor (FGF)-1 on regulation of steady-state PN (A and C) and OPN (B and D) mRNA expression in PASMCs in vitro. Growth-arrested PASMCs were exposed to different concentrations of FGF-1 for 24 h (A and B) or 10 ng/ml FGF-1 for 3, 6, 12, and 24 h (C and D) before being harvested. Controls were PASMCs cultured in 0.1% FBS medium for 24 h and treated with vehicle for an additional 24 h. The mRNA loading was normalized by 18S rRNA. Numbers in parentheses are the number of plates contributing data to each group. Results are means ± SE. *P < 0.05 vs. control group by 1-way ANOVA.

Fig. 4. Dose- and time-dependent effects of ANG II on regulation of steady-state PN (A and C) and OPN (B and D) mRNA expression in PASMCs. Growth-arrested PASMCs were exposed to different concentrations of ANG II for 24 h (A and B) or 100 nM ANG II for 3, 6, 12, and 24 h (C and D) before being harvested. Controls were PASMCs cultured in 0.1% FBS medium for 24 h and treated with vehicle for an additional 24 h. The mRNA loading was normalized by 18S rRNA. Numbers in parentheses are the number of plates contributing data to each group. Results are means ± SE. *P < 0.05 vs. control group by 1-way ANOVA.
RNA isolation reagent (GIBCO BRL, Life Technologies). Northern analysis was performed with 32P-labeled selective cDNA probes for PN and OPN, which had been generated in our laboratory by RT followed by DNA PCR, using lung RNA as the template, as previously described (13). A 32P-labeled 18S rRNA oligonucleotide (5'-ACGGTATCTGATCGTCTTCGAACC-3') was used as the control probe to normalize data. Autoradiographic signals were scanned with an optical densitometer (model GS-670 imaging densitometer, Bio-Rad, Hercules, CA). To estimate steady-state-specific mRNA levels, PN and OPN mRNA-to-18S rRNA ratios were determined by dividing the absorbance corresponding to the specific cDNA probe hybridization by the absorbance corresponding to the 18S rRNA probe hybridization. The results were expressed as the ratios of specific mRNA to 18S RNA.

**Western blot analysis for Akt, GSK3, MEK1/2, and ERK1/2.** To evaluate the effects of FGF-1 and ANG II on activities of the phosphatidylinositol 3-kinase (PI3K)/Akt and the Ras/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathways, as well as selectivity of the PI3K inhibitor LY-294002 and the ERK kinase 1/2 (MEK1/2) inhibitor U-0126, Western blot analyses for phosphorylated Akt and GSK3 (an Akt substrate), as well as phosphorylated MEK1/2 and ERK1/2 (p44/p42 MAPK), were performed with the PhosphoPlus Akt antibody kit and phospho-ERK1/2 pathway sample kit (Cell Signaling Technology, Beverly, MD), respectively, as previously described (13). PASMCs were made quiescent by placing them in medium containing 0.1% FBS for 24 h. Other experimental groups were PASMCs pretreated with the phosphatidylinositol 3-kinase 3-kinase (PI3K) inhibitors LY-294002 (LY; 10 μM) or wortmannin (WM; 20 μM), as well as the MEK1/2 inhibitors U-0126 (5 μM) or PD-98059 (20 μM), for 45 min before addition of FGF-1 (10 ng/ml) (A and B) or ANG II (100 nM) (C and D) to the medium or before being exposed to hypoxia (E and F). An additional 24 h incubation was carried out before harvesting for analyses. The mRNA loading was normalized by 18S RNA. Numbers in parentheses are the number of plates contributing data to each group. Results are means ± SE. *P < 0.05 vs. respective control groups. #P < 0.05 vs. respective FGF-1 or ANG II groups. &P < 0.05 vs. respective vehicle control groups by 1-way ANOVA. Results for rapamycin (100 nM, a p70S6K inhibitor), SB-203580 (SB; 10 μM, a p38 MAPK inhibitor), and SP-600125 (20 μM, a JNK inhibitor) are also shown.
exposed in the linear range of film density were scanned with a densitometer (model GS-670 imaging densitometer, Bio-Rad).

Reagents. ANG II and the ANG II type 1 (AT1) receptor antagonist losartan, human recombinant FGF-1, and transcriptional inhibitor actinomycin D were purchased from Sigma (St. Louis, MO). Selective rabbit anti-PN polyclonal antibody was provided by Drs. Lan Bo Chen and Meiru Dai at Harvard Medical School (26). The FGF-1 receptor tyrosine kinase inhibitor PD-166866 was provided by Parke-Davis Pharmaceutical Research, Division of Pfizer (17). The p38MAP and p70S6 kinase activator anisomycin, the MEK1/2 inhibitors U-0126 and PD-98059, the PI3K inhibitors LY-294002 and wortmannin, the p38MAP kinase inhibitor SB-203580, the p70S6 kinase inhibitor rapamycin, and the JNK inhibitor SP-600125 were purchased from Calbiochem-Novabiochem (San Diego, CA).

Statistical analysis. Results are expressed as means ± SE. Statistical analyses were carried out with the SigmaStat package (Jandel Scientific Software, San Rafael, CA) on a personal computer. Statistical comparisons of mRNA levels were performed with the one-way ANOVA or unpaired t-test. If ANOVA results were significant, a post hoc comparison among groups was performed with the Newman-Keuls test. Differences were reported as significant if the P value was <0.05.

RESULTS

Effects of hypoxia on PN and OPN expression in lungs and PASMCs. Chronic normobaric hypoxic exposure for 2 wk significantly increased steady-state PN (a 2.2-fold increase) and OPN (a 2.7-fold increase) mRNA levels in rat lung (Fig. 1, A and B). Immunohistochemical analysis showed that 2-wk exposure to normobaric hypoxia increased PN expression in pulmonary artery and bronchi of hypoxia-adapted rats compared with air controls (Fig. 2). PN was mainly expressed (brown stain) in medial smooth muscle cells and bronchial epithelial cells. Exposure of growth-arrested PASMCs to 1% O2 (Po2 = 20–30 Torr in culture media) for 6, 12, and 24 h resulted in a slight but significant increase in PN (a 40% increase) mRNA levels at 24 h but had no effect on OPN (Fig. 1, C and D). The effect of hypoxia on PN mRNA expression in PASMCs in vitro was much less robust than that in intact lung in vivo. No significant cell death [assessed by Trypan blue (0.4%) exclusion] was observed in PASMCs exposed to hypoxia or normoxia. These results suggest that other mediators, such as hypoxia-responsive growth factors, may be needed to increase expression of PN and OPN in isolated PASMCs in vitro.

Dose- and time-dependent effects of FGF-1 and ANG II on PN and OPN expression in PASMCs. Quantitative Northern blot analysis demonstrated that PN and OPN mRNA expression increased in a dose-dependent fashion (range of 0.1–20 ng/ml) in PASMCs treated with FGF-1 for 24 h (Fig. 3, A and B). The threshold concentration was 0.1 ng/ml (~6.7 PM), and the largest effect was observed at doses of 10 and 20 ng/ml for PN (a 2.1-fold increase) and OPN (a 7.6-fold increase), respectively. The time course for enhancement of PN and OPN expression with FGF-1 was examined at a concentration of 10 ng/ml. Significant increases in PN and OPN mRNA levels in PASMCs were observed after 3 h of FGF-1 treatment (Fig. 3, C and D). PN and OPN mRNA levels increased progressively over the 24-h period of FGF-1 exposure.

Similarly, PN and OPN mRNA expression increased in a dose-dependent fashion (range of 0.1–100 nM or 0.1–100 ng/ml) in PASMCs treated with ANG II for 24 h (Fig. 4, A and B). The threshold concentration was 0.1 nM (or 0.1 ng/ml), and the largest effect (a 3.2-fold increase of PN and a 6.5-fold increase of OPN) was observed at the maximal dose of 100 nM. The time course for enhancement of PN and OPN gene expression with ANG II was examined at a concentration of 100 nM. Significant increases in PN and OPN mRNA levels in PASMCs were observed after 3 h of ANG II treatment (Fig. 4, C and D).

Effects of receptor inhibition on FGF-1- or ANG II-induced upregulation of PN and OPN expression. PASMCs were pretreated with PD-166866 (5 μM), a potent and selective inhibitor of FGF-1 receptor tyrosine kinase (17), or losartan (10 μM), a selective ANG II AT1-receptor antagonist, for 45 min before FGF-1 (10 ng/ml) or ANG II (100 nM) was added to the medium for an additional 24 h of incubation. PD-166866 or losartan completely blocked FGF-1- or ANG II-induced upregulation of PN and OPN mRNA expression (Fig. 5, A–D), indicating the receptor dependence of growth factor stimulation. FGF-1-induced upregulation of PN and OPN mRNA expression was also completely blocked by pretreatment of PASMCs with actinomycin D (5 μM) (data not shown), indicating that transcription is essential to FGF-1-stimulated PN and OPN mRNA expression.

Effects of signal transduction pathway inhibitors on FGF-1- or ANG II-induced upregulation of PN and OPN expression. PASMCs were pretreated with inhibitors for 45 min before FGF-1 (10 ng/ml) or ANG II (100 nM) was added to the medium for an additional 24 h of incubation. LY-294002 (5 μM) or wortmannin (20 μM), PI3K inhibitors, as well as U-0126 (5 μM) or PD-98058 (20 μM), MEK1/2 inhibitors, completely blocked the FGF-1- or ANG II-induced upregula-
tion of PN mRNA expression (Fig. 5, A and C). The MEK1/2 inhibitors U-0126 (5 μM) and PD-98059 (20 μM) also completely blocked FGF-1- or ANG II-induced upregulation of OPN mRNA expression (Fig. 5, B and D). In contrast, the PI3K inhibitors LY-294002 (5 μM) and wortmannin (20 μM) did not alter the stimulatory effect of FGF-1 or ANG II on OPN mRNA expression.

The effects of signaling pathway inhibitors on PN and OPN mRNA expression in PASMCs exposed to normoxia (control) or hypoxia for 24 h are shown in Fig. 5, E and F. PASMCs were pretreated with inhibitors for 45 min before they were exposed to hypoxia (1% O₂) for an additional 24 h. Inhibition of PI3K (by LY-294002) and p38MAPK kinase (by SB-203580) decreased PN mRNA expression, and inhibition of PI3K (by LY-294002), MEK1/2 (by PD-98059), and JNK (by SP-600125) decreased OPN mRNA expression in PASMCs exposed to either normoxia or hypoxia.

Western blot analysis confirmed that FGF-1 or ANG II significantly increased activation of PI3K and MEK1/2 within 15 min in PASMCs. The FGF-1- or ANG II-stimulated phosphorylation of Akt and GSK3 was blocked by pretreatment with the selective PI3K inhibitor LY-294002 (Fig. 6A), and the FGF-1- or ANG II-stimulated phosphorylation of ERK1/2 was blocked by pretreatment with the selective MEK1/2 inhibitor U-0126 (Fig. 6B). LY-294002 did not block FGF-1- or ANG II-stimulated phosphorylation of ERK1/2 (data not shown), and U-0126 did not block FGF-1- or ANG II-stimulated phosphorylation of Akt (data not shown) or MEK1/2 (Fig. 6B), indicating the selectivity of these signaling pathway inhibitors.

Effects of p70S6K and p38MAPK activation on PN and OPN expression in PASMCs. Quiescent PASMCs were treated with FGF-1 (10 ng/ml) or the p38MAPK and p70S6K activator anisomycin (1 μg/ml) for 24 h with or without pretreatment (45 min) with selective inhibitors of intracellular signaling, includ-
ing rapamycin (100 nM, a p70S6K inhibitor), SB-203580 (10 
µM, a p38MAPK inhibitor), or SP-600125 (20 
µM, a JNK inhibitor). Both FGF-1- and anisomycin-induced PN expression were blocked by p70S6K and p38MAPK inhibitors but not by the JNK inhibitor (Fig. 7, top). In contrast, the FGF-1- 
induced OPN expression was blocked by the JNK inhibitor but not by p70S6K and p38 MAPK inhibitors (Fig. 7, bottom left). Anisomycin alone slightly but significantly decreased OPN expression in PASMCs, and rapamycin, SB-203580, and SP-
600125 did not alter the effects of anisomycin on OPN expression in PASMCs (Fig. 7, bottom right).

**DISCUSSION**

The present study is the first to demonstrate increased 
expression of PN in lung and pulmonary arteries of rats 
adapted to hypoxia as part of a generalized ECM response to 
hypoxic stress, which also included increased OPN mRNA 
expression. We have previously reported that hypoxia induces 
acute pulmonary vasconstriction, chronic pulmonary hyper-
tension, and pulmonary vascular remodeling characterized by 
medial hypertrophy and marked increased medial wall thick-
ness of small pulmonary arteries (50–100 µm) in the rat (3, 4). 
The present results demonstrate localization of hypoxia-in-
duced PN expression to PASMCs of hypertrophic pulmonary 
arteries. This finding, coupled with the observation that PN 
expression increases in parallel with OPN in lung of hypoxia-
adapted rats, suggests an involvement of PN in ECM formation and pulmonary vascular hypertrophy and remodeling in re-
sponse to chronic hypoxia exposure.

Another novel finding of the present study is the demonstra-
tion that the hypoxia-responsive growth factors FGF-1 and 
ANG II stimulate PN expression in PASMCs in vitro. The rapid increases in steady-state PN mRNA levels observed in 
response to low concentrations of FGF-1 and ANG II suggest that these growth factors, in addition to hypoxia per se, could be endogenous physiological stimulators of PN gene expression in PASMCs. The FGFs (at least isoforms FGF-1, -2, and -7) and ANG II are expressed in lung and play important roles in diverse aspects of pulmonary development and growth, 
including epithelial cell, VSMC, and myofibroblast prolifera-
tion, differentiation, angiogenesis, and vascular constriction (14, 19). It has been hypothesized that these growth factors 
may contribute to hyperproliferation of PASMCs and muscu-
larization of the pulmonary vasculature in hypoxia-adapted animals (14, 19).

OPN is abundantly expressed in a range of lung diseases (16, 25). Bronchial epithelial cells and alveolar macrophages in 
normal lung express OPN (2), and this expression is greatly 
amplified in epithelium, alveolar, and interstitial macrophages, 
fibroblasts, and endothelial cells and VSMCs in injured and 
diseased lungs (16). Microarray analysis (7) has demonstrated 
increased OPN mRNA expression in the lungs of rats and mice 
exposed to chronic hypobaric hypoxia for 1 and 3 wk. These 
data provide indirect evidence that OPN may play a role in the 
pathogenesis of pulmonary vascular disease.

The present findings extend previous observations of the 
effects of hypoxia exposure on OPN expression in vivo and in 
vitro by elucidating a role for hypoxia-responsive growth 
factors on this process. Although chronic hypoxia exposure per 
se increased OPN gene expression in lungs of hypoxia-adapted 
rats, FGF-1 and ANG II, but not hypoxia per se, increased 
OPN mRNA expression in PASMCs in vitro. This result is

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**Fig. 8.** A simplified schematic illustration of the specific 
action of various signaling pathway inhibitors and the 
intracellular signaling pathways that mediate FGF-1- or 
ANG II-induced PN and OPN expression in cultured 
PASMCs. RTK, receptor tyrosine kinase; GPCR, G-pro-
tein coupled receptor.
similar to our laboratory’s (22) previous findings that ANP clearance receptor (NPR-C) expression is downregulated in lungs of rats and mice exposed to hypoxia but not in PASMCs cultured under hypoxic conditions. The absence of a direct effect of hypoxia on OPN mRNA levels in PASMCs in vitro differs from the result of Sodhi et al. (20), who found a biphasic increase in OPN mRNA and protein levels with exposure to 3% O₂ (maximal at 2 h, no effect at 6 and 12 h, and slight increase at 24 h) in rat mesangial cells and aortic smooth muscle cells in vitro. In the present study, we did not sample time points earlier than 6 h; however, we found no effect of hypoxia (1% O₂) on OPN expression in PASMCs at 6, 12, or 24 h. Apparent differences between these results may relate to differences in tissues of origin of the smooth muscle cells (kidney/aorta vs. lung), duration of hypoxia exposure, or the severity of hypoxia. The observations that growth factors time and dose dependently induced increases in OPN mRNA expression in PASMCs in vitro and that FGF-1, FGF-2 (13), and ANG II (19) expression increased in lung of rats adapted to chronic hypoxia provide additional evidence for a functional role for hypoxia-responsive growth factors in stimulating ECM formation and remodeling in the hypoxic lung.

FGF-1 binds to any of four receptor isoforms (FGFR-1, -2, -3, and -4), and ANG II binds to at least 2 ANG receptors (AT₁ and AT₂). FGFR-1 and AT₁ are the predominant receptor isoforms in adult VSMCs (1, 27). PD-166866 is a potent (nanomolar range) and highly selective small-molecule inhibitor of FGFR-1-dependent signaling that does not perturb activated PASMC expression of PN and OPN mRNA after FGF-1 or ANG II treatment. This result indicates that FGF-1 or ANG II stimulation of PN or OPN mRNA expression in PASMCs in vitro is mediated in an FGFR-1-dependent or AT₁-dependent manner.

Multiple intracellular signaling pathways are activated by FGFs and ANG II (8, 9) (Fig. 8). Activation of the PI3K-mediated Akt and p70S6K pathways and activation of the Ras-mediated MEK1/2/ERK1/2, p38MAPK, and JNK pathways are necessary for the mitogenic activity of FGFs and ANG II in VSMCs (8, 15, 24). In this study, we investigated the involvement of PI3K-mediated and Ras-mediated signaling pathways in the regulation of PN expression in PASMCs. Inhibition of PI3K by LY-294002 or wortmannin, p70S6K by rapamycin, MEK1/2 by U-0128 or PD-98059, and p38MAPK by SB-203580 significantly inhibited FGF-1- or ANG II-induced PN mRNA expression. However, inhibition of JNK by SP-600125 had no effect on PN mRNA expression under FGF-1- or anisomycin-treated conditions. These results suggest that the PI3K/p70S6K, Ras/MEK1/2/ERK1/2, and Ras/p38MAPK pathways, but not the Ras/JNK pathway, are involved in FGF-1- or ANG II-stimulated PN mRNA upregulation in PASMCs (Fig. 8).

This study elucidates for the first time the signaling pathways by which FGF-1 and ANG II stimulate OPN mRNA expression in PASMCs (Fig. 8). We observed that FGF-1 and ANG II potently and time and dose dependently stimulate expression of OPN mRNA in PASMCs in vitro through the activation of the Ras/MEK1/2/ERK1/2 and Ras/JNK pathways but not the PI3K or p38MAPK signaling pathways. These results are consistent with our previous observation that FGF ligand-activated FGFR-1 plays a significant role in upregulation of OPN gene expression at the transcriptional level via activation of FGFR-1 and subsequent activation of the Src/Ras/MEK/MAPK pathway, but not the PI3K and p38MAPK pathways, in rat aortic smooth muscle cells in vitro (11).

In summary, the present study provides the first evidence that the PN mRNA expression is increased in lung of hypoxia-adapted animals and in cultured PASMCs exposed to hypoxia and that the hypoxia-responsive growth factors FGF-1 and ANG II play significant roles in upregulation of PN and OPN expression at the transcriptional level via activation of FGFR-1 and AT₁ receptors, respectively. We also demonstrated that activation of the PI3K/Akt/p70S6K, Ras/MEK1/2/ERK1/2, and Ras/p38MAPK signaling pathways, but not the Ras/JNK pathway, by FGF-1 or ANG II increases PN gene expression in PASMCs in vitro. In contrast, the FGF-1- and ANG II-stimulated OPN expression in PASMCs is mediated through activation of Ras/MEK1/2/ERK1/2 and Ras/JNK pathways but not the PI3K/Akt/p70S6K or Ras/p38MAPK signaling pathways. These differences in signaling suggest that PN and OPN may play different roles in pulmonary vascular remodeling.

GRANTS

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